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Activation of Silent Replication Origins at Autonomously Replicating Sequence Elements near the *HML* Locus in Budding Yeast

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In the budding yeast, *Saccharomyces cerevisiae*, replicators can function outside the chromosome as autonomously replicating sequence (ARS) elements; however, within chromosome III, certain ARSs near the transcriptionally silent *HML* locus show no replication origin activity. Two of these ARSs comprise the transcriptional silencers E (*ARS301*) and I (*ARS302*). Another, *ARS303*, resides between *HML* and the *CHA1* gene, and its function is not known. Here we further localized and characterized *ARS303* and in the process discovered a new ARS, *ARS320*. Both *ARS303* and *ARS320* are competent as chromosomal replication origins since origin activity was seen when they were inserted at a different position in chromosome III. However, at their native locations, where the two ARSs are in a cluster with *ARS302*, the I silencer, no replication origin activity was detected regardless of yeast mating type, special growth conditions that induce the transcriptionally repressed *CHA1* gene, *trans*-acting mutations that abrogate transcriptional silencing at *HML* (*sir3*, *orc5*), or *cis*-acting mutations that delete the E and I silencers containing ARS elements. These results suggest that, for the *HML* ARS cluster (*ARS303*, *ARS320*, and *ARS302*), inactivity of origins is independent of local transcriptional silencing, even though origins and silencers share key *cis*- and *trans*-acting components. Surprisingly, deletion of active replication origins located 25 kb (ORI305) and 59 kb (ORI306) away led to detection of replication origin function at the *HML* ARS cluster, as well as at *ARS301*, the E silencer. Thus, replication origin silencing at *HML* ARSs is mediated by active replication origins residing at long distances from *HML* in the chromosome. The distal active origins are known to fire early in S phase, and we propose that their inactivation delays replication fork arrival at *HML*, providing additional time for *HML* ARSs to fire as origins.

In eukaryotic chromosomes, duplication of genetic information occurs during the S phase of the cell cycle and is coordinately regulated with the separation of the sister chromatids in mitosis (45). A chromosome initiates duplication at multiple DNA replication origins, and each origin is regulated to fire only once per S phase (16, 26, 47). Timing of initiation is also regulated within S phase, and DNA replication origins fire in a characteristic order (22). The temporal firing order often correlates with transcriptional activity: early-replicating regions of chromosomes are associated with active genes, and late-replicating regions are associated with silent genes (27, 28). Genetic elements that activate transcription are sometimes closely associated with active replication origins (15). Conversely, genetic elements that silence transcription are in some cases intimately associated with silent replication origins. It is not understood how DNA replication origins are silenced in chromosomes.

Genetic elements that function in *cis* to activate a DNA replication origin comprise the replicator. In the budding yeast, *Saccharomyces cerevisiae*, replicators can be isolated as genomic fragments that function as autonomous replicating sequence (ARS) elements in plasmids (64). ARS elements share a number of essential and important *cis*-acting determinants with the chromosomal replicators from which they were derived (18, 31, 32, 40, 57, 66). However, it remains puzzling why certain ARSs that mediate DNA replication in a plasmid are not detectably active as replication origins within the chromosome.

A number of ARS elements have been suggested to be inactive as replication origins in *S. cerevisiae* chromosome III (20, 54). Several such ARSs map near the *HML* locus, a transcriptionally silent mating-type locus on the left arm of the chromosome. *ARS301* and *ARS302* function in silencing transcription at *HML* (see below). *ARS303* maps near *ARS302* but is not essential for transcriptional silencing. Additional ARSs that are not detectably active as replication origins in the chromosome map at other locations, including near the transcriptionally active mating-type locus, *MAT* (48). The nature of the determinants responsible for the inactivity of replication origin function at *HML* ARSs in the chromosome is presently unknown.

Certain *HML* ARS elements are intimately associated with *cis*-acting elements called transcriptional silencers (7, 8). *HML* silencers E and I correspond to *ARS301* and *ARS302*, respectively (39, 48). An ARS consensus sequence which interacts with the six-subunit origin recognition complex (ORC) and is essential for the initiation of DNA replication at active origins also has a role in maintenance of transcriptional repression via ORC binding at the silencer elements (2, 24, 42). In addition, binding sites for Rap1p and Abf1p, which stimulate replication initiation at some ARSs (41), contribute to ARS-mediated transcriptional silencing (11, 61). Four silent information regulator proteins, Sir1p to Sir4p, are essential for transcriptional repression (55), and they mediate interactions among ORC, Rap1p, and histones H3 and H4 to form a heterochromatin-like structure (29, 67, 68). Transcriptional silencers that flank the *HMR* locus on the right arm of chromosome III also contain ARSs but, unlike the ARSs at *HML*, *HMR* ARSs are active as replication origins in the chromosome (56, 57). At *HML*, all of the ARSs have been suggested to be inactive as origins (20). A pre-replicative complex (pre-RC) involving ORC can form at *ARS301*, the *HML* E silencer (58). This presents a paradox since the existence of a pre-RC is thought to reflect a

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potentially active replication origin, yet *ARS301* is inactive as an origin.

The relationship between transcriptional silencing and inactive replication origins at *HML* ARSs has not been extensively studied. Strains with *sir1* or *sir4* mutations that relieved transcriptional repression showed no detectable initiation of replication from *ARS301* and *ARS302*, the E and I silencers (20). The large number of genes involved in the transcriptional silencing process and the fact that the process shares key *cis*- and *trans*-acting components involved in the activation of replication origins indicates that further investigation is needed before firm conclusions can be drawn about why replication origins are inactive at *HML*. Mutations in *SIR3* have not been examined but may be relevant since Sir3p has some unique features, including structural similarities with Orc1p and Cdc6p which are required for replication initiation (3) and an enhanced ability to propagate silenced chromatin (12, 53). Importantly, no *cis*-acting mutations that eliminate the transcriptional silencers (38) and no *trans*-acting mutations that selectively disrupt the transcriptional silencing function of ORC (19, 25) have been examined for their effects on replication origin activity at *HML* ARSs.

Other hypotheses have been considered to account for the inactivity of *HML* ARSs as chromosomal replication origins. One hypothesis is that some aspect of chromosome context, such as proximity to telomeres, is important. Telomeres are determinants of late replication timing at certain origins (23), and the *HML* ARSs are within 10 to 15 kb of the left telomere in chromosome III. However, no activity of *HML* ARSs was detected in a circular chromosome III derivative that lacked telomeres (20). Also, a strain containing an insertion of *HML ARS301* near the *MAT* locus far from the telomere showed no replication origin activity at that ARS. Another hypothesis is that *HML* ARSs are specialized replication origins that are active in some special growth conditions or stage in the yeast life cycle (21). There is as yet no support for this hypothesis, but only one study has been performed. In meiosis, *HML* ARSs tested which have no origin activity in mitotic S phase are also not detectably active in premeiotic S phase (14).

Yeast mating type has marked effects on the left arm of chromosome III containing *HML* in terms of DNA recombination competence and chromatin structure (69, 70). *MAT***a** cells activate the entire left arm of chromosome III, while *MAT*α cells inactivate the left arm, including *HML*. The effect of mating type in isogenic strains on replication origin activity at *HML* ARSs has not been previously examined. Certain *HML* ARSs reside near *CHA1* (catabolism of hydroxy amino acids), whose expression is highly inducible in special growth conditions (5, 52, 65). *CHA1* is normally transcriptionally repressed, but in special growth conditions that induce gene expression, a repressive chromatin structure present over the gene promoter region is disrupted (44). The hypothesis that certain *HML* ARSs are specialized replication origins that function only in growth conditions that induce *CHA1* gene expression and open chromatin structure has not been tested.

ARS303 resides in a 1.4-kb region between *HML* and the *CHA1* gene, and its function is not known. Sequences containing *ARS303* are not required for *HML* transcriptional silencing. Unambiguous assessment of replication origin activity associated with *ARS303* was previously not possible since the precise location of the ARS in the 1.4-kb region was unknown. Here we further localized and characterized *ARS303* and found that it is near *ARS302*, the I transcriptional silencer, and in the process we discovered that it is closely associated with a new ARS, *ARS320*. We show that both *ARS303* and *ARS320* are competent as replication origins when inserted at a different location in chromosome III. At their native location, however, where both ARSs are closely clustered with *ARS302*, no replication origin activity is detected independent of yeast mating type, special growth conditions that induce the transcriptionally repressed *CHA1* gene, *trans*-acting mutations that abrogate transcriptional silencing at *HML* (*sir3*, *orc5*), or *cis*-acting mutations that delete the E and I silencers (*ARS301* and *ARS302*). Surprisingly, deletion of active replication origins at remote locations in the chromosome results in replication origin activity at the *HML* ARS cluster. Also, origin activity is seen at *HML ARS301*, previously known to form a pre-RC (58), but thought to be inactive as a replication origin. Our results show that replication origin silencing at *HML* ARSs is mediated by active replication origins located at long distances from *HML*. The distal active origins are known to fire early in S phase (54), and we propose that their deletion delays fork arrival at *HML*, providing additional time for *HML* ARSs to fire as origins.

MATERIALS AND METHODS

Reagents. Restriction enzymes, T4 DNA ligase, Klenow fragment, and T4 DNA polymerase were obtained from New England BioLabs, Inc. [α-^{32}P]dATP was purchased from Amersham International. Medium reagents were from Difco Laboratories and American Biorganics, Inc. 5-Fluoro-orotic acid (FOA) was from Toronto Research Chemicals, Inc. All other chemicals were purchased from Sigma Chemical Company.

Bacteria, plasmids, and yeast. The *Escherichia coli* strain used for transformation and plasmid propagation was DH5α (BRL). The haploid *Saccharomyces cerevisiae* strain used for integrative transformation and the plasmid loss assay was YPH98 (*MAT***a** *ade2-101 lys2-801 ura3-52 trp1-1 leu2-1*) and was obtained from Philip Hieter (Johns Hopkins University). The following *S. cerevisiae* strains, obtained from James Broach (Princeton University), were used for two-dimensional (2-D) gel analysis: DMY1 (*HML*α *MAT***a** *ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100*), DMY2 (DMY1; *sir3*::*LEU2*), DMY94 (DMY1; E$^+$::*URA3* I$^-$Δ242), and DMY95 (DMY1; E$^-$Δ79-113::*URA3* I$^-$Δ242) (38). *S. cerevisiae* JRY4554 (W303-1A; *MAT*α *orc5-1,2 HMR-SS*) and JRY4556 (W303-1A; *MAT*α *orc5-1,3 HMR-SS*) (W303-1A = *MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) (24), obtained from Jasper Rine (University of California, Berkeley), were also used in 2-D gel analysis. *CHA1* gene induction experiments were done in strains X2180-A (*MAT***a** *SUC2 mal mel gal2 CUP1*) and X2180-B (*MAT*α *SUC2 mal mel gal2 CUP1*), which were purchased from the Yeast Genetic Stock Center (University of California, Berkeley). YIp5 plasmids containing DNA fragments of yeast chromosome III (49) were gifts from Carol Newlon (UMD-New Jersey Medical School). The yeast strain with the ORI305 replicator deleted, Δ305, is a derivative of the YPH98 strain (YRH80/433) and was constructed as previously described (31). The yeast strain with both ORI305 and ORI306 replicators deleted, Δ305/Δ306, was obtained by transplacement of pARSΔ306 into homologous sequences near *ARS306* in the Δ305 strain by using methods previously described (31). Plasmid pARSΔ306 was constructed by M. L. He by deleting a 220-bp *Hin*dIII/*Bgl*II fragment containing *ARS306* (18) from pARS306. The latter plasmid is a YIp5 derivative (*URA3* selectable marker) which contains *ARS306* in a 1.2-kb *Eco*RI-*Bsr*GI fragment of the chromosome III C1G region (49). Yeast transformation was performed with *Xho*I-linearized pARSΔ306, and integrants were selected after growth on synthetic medium in the absence of uracil (see below). Homologous recombinants that evicted the integrated plasmid sequences were obtained after selection against *URA3* by growth on medium containing FOA (31). The correct homologous recombinants harboring a deletion at the ORI306 replicator in the chromosome were identified by restriction enzyme mapping after Southern blotting. The absence of origin activity at the Δ306 locus was confirmed by 2-D gel electrophoresis of replication intermediates (see below).

Bacteria were grown at 37°C, in Luria-Bertani medium supplemented with ampicillin (50 μg/ml). Plasmid DNA was obtained from *E. coli* DH5α cells by the boiling method, and minipreps of yeast genomic DNA were carried out as previously described (31). Yeast cells were grown at 30°C either in complete medium, yeast-peptone-dextrose (YPD), or in synthetic dextrose minimal medium (SD) containing supplements (see below) as described by Sherman et al. (59). SD was supplemented with adenine sulfate (20 mg/liter), lysine (30 mg/liter), leucine (30 mg/liter), and tryptophan (20 mg/liter), and this was called supplemented minimal medium (SMM). For the FOA selection process, SMM-agar plates were further supplemented with uracil (50 mg/liter) and FOA (1 g/liter).

Linker substitution mutations by PCR. Linker substitution mutations in *ARS303* and *ARS320* were generated by using two mutant primers for each consensus sequence, one on the top and one on the bottom strand. Mutant primers contained a *Kpn*I sequence (lower cased below) positioned so that it would substitute most of the potential ARS consensus sequence whose function was being tested. The sequences of mutant primers were as follows: 303KL1, 5′-TC

GGCAgggtacccATACCTTAGATGTTACCAGCTGGGAA-3′; 303KL1op, 5′-AGGTATgggtacccTGCCGACATTTGCAGCTCTTTCATCA-3′; 320KL2, 5′-CCTATAgggtacccTATGTTATGTTATGTTTGCTATGACT-3′; and 320KL2op, 5′-AACATAgggtacccTATAGGTTGTACTTTGTCAATAGAAA-3′. Two outside primers were also used that correspond to the *Hin*dIII and *Pst*I restriction sites of the *Hin*dIII/*Bam*HI restriction fragment (see Fig. 1). In each mutagenesis, mutant primers were used with corresponding outside primer to generate two DNA fragments, each with a newly introduced *Kpn*I restriction site on one end. Each PCR reaction was performed by using 8 U of AmpliTaq polymerase (Perkin-Elmer), a deoxynucleoside triphosphate mix in a final concentration of 0.25 mM, 10 ng of template DNA, and 10 pmol of each primer. YIp5 vector with *Hin*dIII/*Bam*HI fragment containing *ARS303*/*ARS320* (see Fig. 1, map) was used as template DNA. After PCR reaction, products were cut with *Kpn*I and the appropriate restriction enzyme for the other end (*Hin*dIII or *Pst*I), ligated together (via the *Kpn*I site), and cloned back into YIp5 or pVHA vectors. Correct plasmid constructs were confirmed by restriction enzyme mapping of the DNA. The DNA sequence of the insert was determined to confirm the mutations and to verify that no undesired mutations were introduced by PCR.

High-frequency transformation (HFT) assay. For the purpose of determining whether a particular fragment has ARS activity, several different fragments (see Fig. 1) were subcloned into the YIp5 vector in appropriate restriction sites. The resulting plasmids were transformed into yeast strain YPH98 by using a lithium-acetate procedure and plated on selective medium (SMM with no uracil added) as described earlier (31). The resulting transformants were scored as Ars^+ if they could be passaged after being streaked on additional selective plates and grown in selective liquid medium.

Mitotic stability and plasmid loss rate assays. For these assays, different ARS derivatives were cloned in a centromere-containing vector, pVHA, and transformed into the YPH98 strain. Assays were performed as described previously (31) but with some modifications. Single transformants were inoculated in 3 ml of selective, liquid minimal medium (SMM with no uracil) and grown for 22 h on 30°C. Cultures were plated on nonselective (YPD) and selective plates (SMM with no uracil) at dilutions that gave rise to ~200 colonies on nonselective plates, and the initial percentage of plasmid-containing cells (I) under selection was determined. The same cultures were also used to inoculate 3 ml of nonselective, YPD medium, at 10^4 cells/ml. Cultures were grown for 12 generations at 30°C and plated on nonselective and selective plates as described above to determine the final percentage of plasmid-containing cells (F) after growth in the absence of selection for a specified number of generations (N). For transformants with extremely slow growth rates, twice and 200 times the amount of cells were plated on the selective plates to determine the I and F values, respectively. Mitotic stabilities (the percentage of plasmid-containing cells) before release of selection and after growth in nonselective medium correspond to I and F, respectively. The rate of plasmid loss per generation is expressed as $1 - (F/I)^{1/N}$.

Transplacement of *HML* ARS sequences into a different location of chromosome III. For the transplacement of *ARS303*, *ARS320*, or both to the chromosomal location of ORI305, a chimeric plasmid was made by inserting an appropriate fragment (343-bp *Pvu*II fragment for *ARS303*, 1,061-bp *Pvu*II/*Bam*HI fragment for *ARS320*, and 1,425-bp *Hin*dIII/*Bam*HI fragment for *ARS303*/*ARS320*) in place of the ORI305 replicator between the *Sac*I and *Cla*I sites of plasmid p305BP (31). After the *Sac*I/*Cla*I restriction digest of p305BP, the larger fragment containing the vector was gel separated from the 335-bp fragment containing the ARS element and, after treatment with T4-DNA polymerase (producing blunt ends), used in a ligation reaction with each of three fragments described above. After identification with the appropriate restriction enzymes, correct chimeric plasmids were linearized within sequences flanking the ORI305 replicator to transplace *HML* ARSs into the Δ305 locus by integrative transformation and homologous recombination as previously described (31). The desired homologous recombinants were identified by the correct size restriction fragments seen after Southern blotting and hybridization with a ^{32}P-labeled DNA probe specific for the region of interest.

2-D gel electrophoresis of DNA replication intermediates. Replication intermediates from yeast cells were isolated as previously described (33). Total genomic DNA was isolated from exponentially growing cultures containing approximately 2×10^7 cells/ml and, after purification on by equilibrium sedimentation in a CsCl density gradient, were digested with appropriate restriction enzyme(s). The replication intermediates were enriched by binding them to a benzoylated, naphthoylated DEAE-cellulose column and elution with 1.8% solution of caffeine. The replication intermediates in the caffeine eluant were analyzed by 2-D gel electrophoresis (10) with minor modifications. In the first-dimension gel, electrophoresis was carried out for 22 h at 1 V/cm in 0.4% agarose in Tris-acetate-EDTA buffer containing 0.1 μg of ethidium bromide per ml. Approximately 8- to 10-cm-long lanes were cut from the first-dimension gel and transferred into the second-dimension gel, which consisted of 1% agarose in Tris-borate-EDTA buffer with 0.5 μg of ethidium bromide per ml. Second-dimension gels were run for 7 to 9 h at 4.5 V/cm at 4°C. The DNA was transferred from the gel to a nylon membrane (GeneScreen Plus; DuPont) by using a pressure blotter (Stratagene), and hybridizations with a ^{32}P-labeled DNA probe specific for the region of interest were carried out as described earlier (39a). Radioactive signals were detected by using a PhosphorImager (Molecular Dynamics).

RESULTS

The map at the top of Fig. 1 shows part of the left arm of chromosome III with all of the ARS elements known prior to this work, as well as some landmarks, including a transcriptionally silent mating-type locus, *HML*, and the left telomere. Of the six ARS elements shown (numbered 300 to 305), only one, *ARS305*, maps physically with origin activity in the chromosome (14, 34, 49). *ARS305* contains functional components of a chromosomal replication origin, ORI305 (31, 32). Other replication origins in the left arm of chromosome III, such as ORI306 and ORI307, also map physically with ARS elements and require ARS components for chromosomal origin function (18, 66, 71). To understand why ARSs localized near *HML* are inactive as chromosomal replication origins, we first localized *ARS303* and then examined its replication activity in the chromosome under different experimental conditions and chromosomal contexts.

Localization of *ARS303* and discovery of a new ARS element near *HML*. *ARS303* was originally defined as a 1.4-kb *Hin*dIII-*Bam*HI chromosomal fragment, which is Ars^+ in the HFT assay and which maps near the *HML* locus (49). Since the size of some ARS elements in *S. cerevisiae* can be reduced to ~0.1 to 0.2 kb with retention of full function (43), we performed a series of subcloning and mutational analyses to further localize *cis*-acting genetic elements responsible for ARS activity within the original clone.

Three different, overlapping fragments, *Hin*dIII-*Bam*HI (H-B; 1,425 bp), *Eco*RV-*Bam*HI (RV-B; 851 bp), and *Hin*dIII-*Pst*I (H-P; 734 bp) were subcloned into the plasmid pVHA (Fig. 1, maps). All three plasmids were tested for the ability to provide HFT of a Ura^- yeast strain (YPH98) under selection for the *URA3* marker in the plasmid. As shown in Fig. 1 in the column labeled "ARS," the fragments H-B and H-P are Ars^+ in pVHA and the RV-B fragment is Ars^-. These data further localize ARS activity within the 734-bp H-P fragment.

Computer analysis of the DNA sequence of the H-P fragment revealed many matches to the ARS consensus sequence (ACS) (11/11, 10/11, or 9/11). The occurrence of a broad easily unwound region, which is 3′ to the T-rich strand of the ACS, has been demonstrated for several ARS elements in *S. cerevisiae* (46). A combined search for both features (ACS match and a low free energy of unwinding 3′ to the ACS T-rich strand [45a]) suggested two matches to the ACS to be candidates for the essential ACS: one on the bottom strand, starting from position 346, and one on the top strand, starting from position 448 of the H-P fragment. These two potential ACSs were individually mutated by replacing them with a *Kpn*I linker (KL1 and KL2; Fig. 1). When introduced separately, none of the mutations abolished the ARS activity of the H-P fragment. Surprisingly, only simultaneous linker substitution of both consensus sequences completely abolished ARS activity (Fig. 1). This result, together with the fact that two consensus sequences are in a tail-to-tail orientation, strongly suggested the presence of two independent ARS elements within the analyzed fragment.

Two ARS elements were identified by additional subcloning. Nonoverlapping fragments, *Pvu*II-*Pvu*II (Pv-Pv; 343 bp) and *Pvu*II-*Bam*HI (Pv-B; 1,061 bp) inserted individually into the pVHA vector each provided HFT of yeast cells, confirming the existence of two independent ARS elements within the originally cloned H-B fragment. We assigned the previously existing name, *ARS303*, to the ARS element with the ACS 5′-ATTTATATTTT-3′ in the bottom (minus) strand. We designated the new ARS element with the ACS 5′-TTTTATGTTAT-3′ in the top (plus) strand as *ARS320* (Fig. 1). The *ARS303* essential

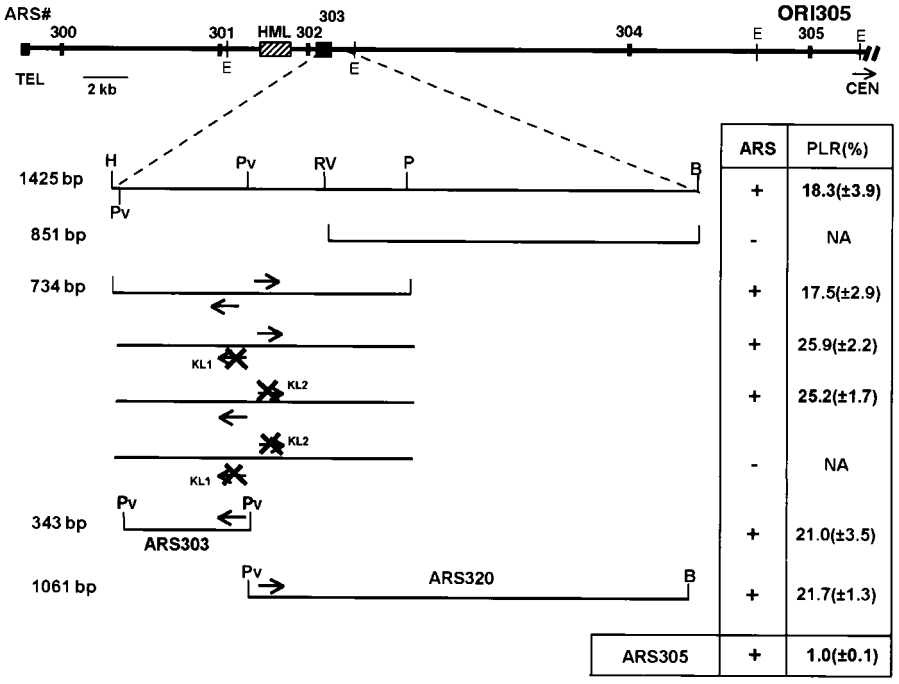

FIG. 1. Localization of *ARS303* and discovery of *ARS320*. Subcloning and mutational analysis were used in localization of *ARS303*. The top diagram shows a map of the first 40 kb of the left arm of chromosome III drawn to scale by using DNA sequence information (50). On the map are previously known ARS elements (numbered 300 to 305), the silent mating-type locus *HML*, the left telomere, and some relevant restriction sites. The *Hin*dIII/*Bam*HI fragment (1,425 bp) is blown up below the chromosome III map, with relevant restriction sites and potential ARS consensus sequences (arrows) shown. Introduced *Kpn*I linkers (KL1 and KL2) that disrupt two potential ACSs are shown as a crossed arrows. The ARS function of each subclone is indicated as "+" for active ARS derivatives or "−" for inactive ARS derivatives. Percent PLR determinations are average values from at least three different experiments. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Pv, *Pvu*II; RV, *Eco*RV; P, *Pst*I.

sequence resides in a perfect match to the ACS, and in *ARS320* the essential sequence it resides in a 10/11 match with overlapping 9/11 matches. No ARS activity was detected when the same linker substitutions of these ACS matches were tested in the separated ARS elements (data not shown), confirming an essential role for the substituted sequences in plasmid replication.

A quantitative plasmid loss rate (PLR) assay was performed on the ARS$^+$ derivatives to assess their replication efficiency. The assay is independent of possible effects of the ARS element on plasmid segregation or on selectable marker expression since the pVHA vector contains a centromere to promote proper segregation and the assay is done in the absence of selection for the *URA3* marker. The lower the PLR, the higher the replication efficiency of the ARS. As seen in Fig. 1 in the column labeled PLR, all of the ARS derivatives tested have a high PLR compared to an efficient replicator, *ARS305*. Thus, ARS function is inefficient for the separated *ARS303* and *ARS320* elements, as well as for the various restriction fragments or mutant derivatives of the two ARS elements tested (Fig. 1). Attempts to further localize *ARS320* indicated that the *Pvu*II-*Pst*I fragment was Ars$^+$, but the PLR was not determined.

Our subcloning and mutational analyses of *ARS303* served to localize the ARS element and to define the essential ACS. The analyses also revealed the existence of a new, independent ARS element, *ARS320*, within the originally isolated 1.4-kb Ars$^+$ fragment. While certain other ARSs are known to be closely spaced (35, 60), *ARS303* and *ARS320* are the most closely spaced ARS elements identified so far in *S. cerevisiae* chromosomes. Their essential ACS elements are only 102 bp apart. On average, ARSs are spaced every 14 kb in chromosome III (48). *ARS303* and *ARS320*, together with *ARS302* (Fig. 1, map at top), form a dense cluster of three ARSs in ~1 kb of DNA. We refer to this region as the *HML* ARS cluster. The possible functional significance of this unusual clustering of ARS elements is presently unclear.

HML* ARS elements *ARS303* and *ARS320* are inactive as replication origins in the chromosome independent of mating type.** Precise localization of essential *cis*-acting elements of *ARS303* and *ARS320* enabled us to optimize detection of potential replication origin activity associated with these ARSs in the chromosome. Origin firing creates replication bubbles whose detection by 2-D gel electrophoretic analysis is favored by positioning the essential core of the ARS in the central region of the restriction fragment. Therefore, we examined the origin activity at the *HML* locus by using a restriction enzyme digest that would appropriately center the essential core of *ARS303* and the newly discovered *ARS320* within the fragment used in 2-D gel analysis. Yeast mating type has profound effects on the left arm of chromosome III in terms of DNA recombination competence and chromatin structure (69, 70). *MAT*α cells inhibit recombination along the entire left arm, while *MATa** cells activate recombination in the same region (70). The effect of mating type in isogenic strains has not been examined previously. Thus, we examined the two yeast mating types in isogenic strains for replication origin activity near *HML*.

Replication bubbles resulting from initiation of DNA replication within the restriction fragment analyzed are detected as a high rising arc (bubble arc) after neutral-neutral 2-D gel electrophoresis (10) and probing with appropriate DNA probe (Fig. 2A, Ori$^+$). Passive replication originating outside of the restriction fragment analyzed results in fork-shaped molecules with a single replication fork, and these are detected as a distinct Y-arc (Fig. 2A, Ori$^-$).

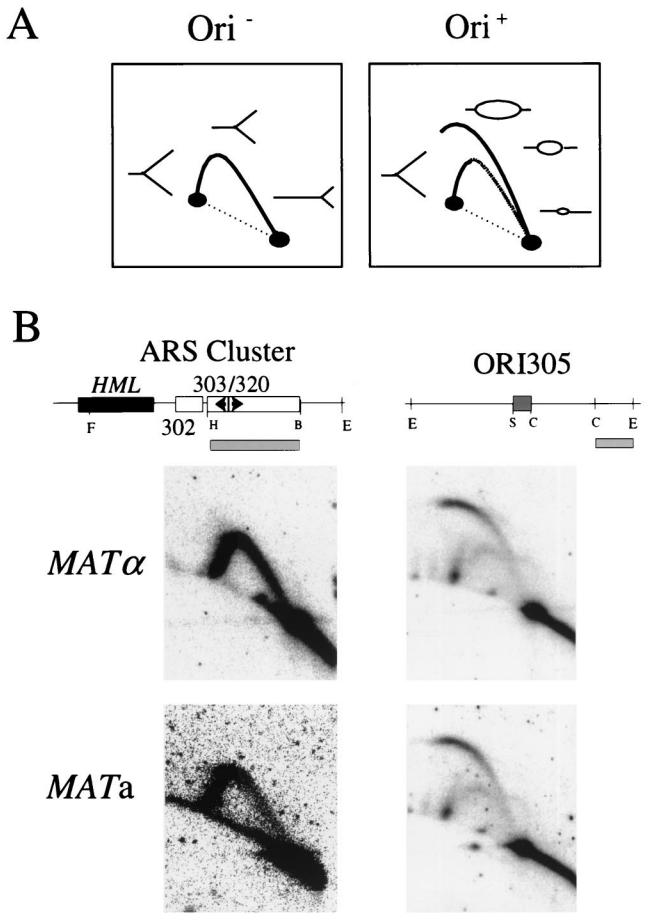

FIG. 2. Replication origin activity is not detectable at the *HML* ARS cluster in *MAT*α or *MAT***a** cells. The *HML* ARS cluster containing *ARS302*, *ARS303*, and *ARS320* was analyzed by 2-D gel electrophoresis in both mating types of *S. cerevisiae* X2180, *MAT*α and *MAT***a**. (A) Schematic drawing of replication intermediates that can be detected when analyzed restriction fragment is passively replicated by forks coming from the outside (Ori^-) or when an active replication origin is centrally located within the analyzed fragment (Ori^+). The arc in the Ori^- example is called a Y arc. The right half of the arc is the early Y arc, and the left half is the late Y arc. The high rising arc in the Ori^+ example is called a bubble arc. (B) 2-D gel analysis of replication intermediates from the *HML* region of chromosome III (left). The map above shows a portion of the left end of chromosome III (ca. 4.5 kb) containing the *HML* locus and the *ARS302*/*ARS303*/*ARS320* region, with relevant restriction sites. The probe used in this analysis is shown as a shaded box below the map. *Fsp*I/*Eco*RI genomic digest produces a 3.9-kb fragment with the three ARS elements positioned in a central region to favor detection of replication bubbles. As a positive control, the same blot was reprobed for ORI305. The map above shows the *Eco*RI fragment of chromosome III containing ORI305 with relevant restriction sites and the probe used in this analysis (shaded box).

*Fsp*I and *Eco*RI digestions produce a 3.9-kb fragment at the *HML* locus, with the ACS elements of *ARS303* and *ARS320* within the central region of the fragment (Fig. 2B, arrows in left map). This fragment overlaps the *HML* ARS cluster, and so it includes *ARS302* which has been suggested to be nonfunctional as a chromosomal origin (20). Even in a restriction fragment chosen to optimize detection of potential replication bubbles, no bubble arc was seen after 2-D gel analysis (Fig. 2, *MAT*α, left panel). In order to test whether this result is due to artifactual loss of replication bubbles caused by breakage, the same blot was reprobed to detect ORI305, which is an efficient replication origin (31). An *Fsp*I/*Eco*RI genomic digest produces 5.8-kb *Eco*RI/*Eco*RI fragment containing ORI305 in a central position (Fig. 2B, right map). Abundant replication bubbles are detected at ORI305 (Fig. 2, *MAT*α, right panel), indicating that the absence of detectable replication bubbles at the *HML* locus in the *MAT*α strain is not due to extensive losses caused by breakage. The results extend the findings of Dubey et al. (20) for *ARS302* to a different *MAT*α strain. We conclude that in the *MAT*α strain analyzed no ARSs in the *HML* ARS cluster (*ARS303*, *ARS320*, and *ARS302*) are detectably active as chromosomal replication origins.

In addition, the same 2-D gel analysis was performed in an isogenic strain with the opposite mating type, *MAT***a**. No replication initiation could be detected at the *HML* ARS cluster (Fig. 2, *MAT***a**, left panel), while efficient initiation was detected at ORI305 (Fig. 2, *MAT***a**, right panel). These results indicate that *ARS303*, *ARS320*, and *ARS302* are not detectably active as chromosomal replication origins in the isogenic *MAT***a** strain. Each mating type of another pair of isogenic yeast strains (YPH98 and YPH102) was also analyzed, with the same results (data not shown). The persistence of origin inactivity independent of the mating type in isogenic strains suggests that inactivity of these ARSs as chromosomal replication origins does not depend on properties of the left arm of chromosome III, such as differences in DNA recombination competence or chromatin structure known to be specified by the two different mating types of yeast cells (69, 70).

***HML* ARS elements *ARS303* and *ARS320* are active as replication origins when inserted at a different location in the chromosome.** It is not known whether any *HML* ARS is competent to function as a chromosomal origin outside their native location near *HML*. In order to test this, we analyzed the replication origin activity of copies of *ARS303* and *ARS320* transplaced into a different location in chromosome III. *ARS303* and *ARS320* were inserted in place of the ORI305 replicator (Fig. 3A). A variety of mutations that affect ORI305 activity at this chromosomal locus have been previously characterized (31, 32). A DNA region larger than ones known to be sufficient for ORI305 activity in the chromosome was deleted (Fig. 3A, Δ305). This chromosomal locus was chosen for the transplacement of these *HML* ARSs for two primary reasons. First, deletion of DNA regions smaller than that in Δ305, and even point mutations in the essential ACS of the replicator, have been shown to completely abolish ORI305 activity in the chromosome (31). Thus, the Δ305 locus has no cryptic origin function. Second, the locus is permissive for origin function, not only for ORI305 but also for active origins from other yeast chromosomes (42a). Therefore, insertion of potential replicators into the Δ305 locus avoids any negative effects on origin function that might be encountered after transplacement into an uncharacterized chromosomal location.

Three new strains with *ARS303*, *ARS320*, or *ARS303*/*ARS320* in place of the ORI305 replicator in the chromosome were confirmed by Southern blot analysis (Fig. 3B, maps, and data not shown). Chromosomal origin activity at the Δ305 region in these three strains was analyzed by 2-D gel electrophoresis. As seen in Fig. 3B (2-D gel panels), bubble arcs, indicative of active replication origins, were detected in all three strains. The data show that *ARS303* and *ARS320*, when present individually or together in their natural closely spaced configuration, have the ability to initiate DNA replication in the chromosomal context of the Δ305 locus.

Our data show for the first time that two ARSs, *ARS303* and *ARS320*, which are inactive as replication origins at their native loci near *HML*, can function as replication origins when transplaced into a different location of the same chromosome. These findings suggest, as one possibility, that replication origin activity of *ARS303* and *ARS320* at the *HML* locus is somehow silenced.

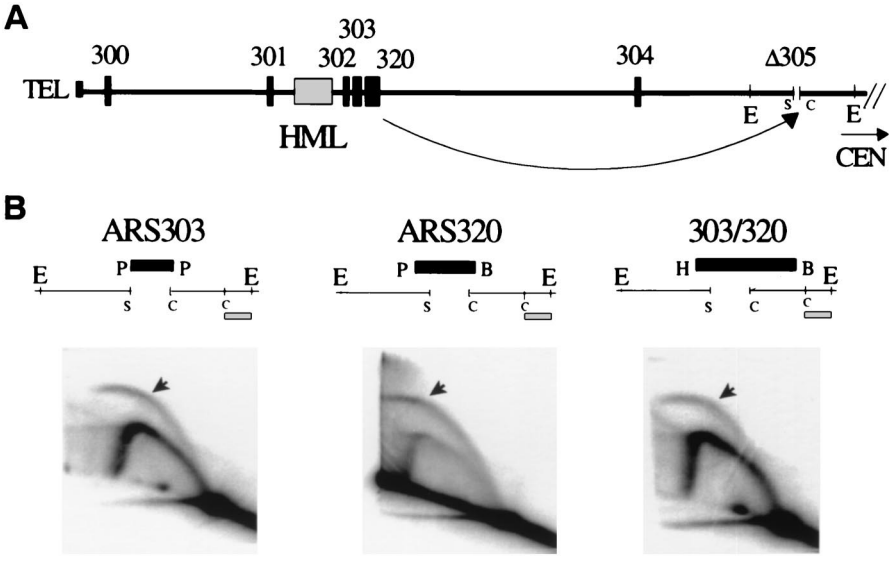

FIG. 3. *HML ARS303* and *ARS320* are active as replication origins when inserted at a different location in the chromosome. (A) The map shows first 40 kb of the left arm of chromosome III with native locations of *HML* ARSs, as well as the position of their transplacement at the location of inactive ORI305 (Δ305). (B) Maps above each 2-D gel show the details of the transplacement in each strain: *ARS303*-containing fragment (*Pvu*II/*Pvu*II, 343 bp) (left), *ARS320*-containing fragment (*Pvu*II/*Bam*HI, 1,061 bp) (middle), or *ARS303*/*ARS320*-containing fragment (*Hin*dIII/*Bam*HI, 1,425 bp) (right) were transplaced into the Δ305 locus between *Sac*I and *Cla*I restriction sites. Replication origin activity of *ARS303*, *ARS320*, and *ARS303*/*ARS320* were analyzed by 2-D gel electrophoresis. *Eco*RI genomic digest (producing 5.8-kb fragment for *ARS303* transplacement, 6.5-kb fragment for *ARS320* transplacement, and 6.9-kb for *ARS303*/*ARS320* transplacement) was used for the 2-D gel analysis. The *Cla*I/*Eco*RI fragment was used as a probe (indicated as hatched box below each map).

Inactivity of the *HML* ARS cluster is independent of *cis* elements and *trans*-acting factors that silence transcription. The existence of common functional elements between transcriptional silencing and replication initiation suggests a functional interdependence between the two processes. We examined the effects of mutations in certain *cis* elements and *trans*-acting factors that participate in transcriptional silencing for their effects on replication origin activity at the *HML* ARS cluster.

Deletions that removed the *cis*-acting silencer elements E (*ARS301*) and I (*ARS302*) were tested. These elements bind several proteins, including ORC, Rap1p, and Abf1p, that contribute to transcriptional silencing at *HML* and to replication initiation in other chromosomal contexts. The silencer elements also contribute to establishing a heterochromatin-like structure at and near *HML*. Deletion of both the E and I elements abrogates transcriptional silencing at *HML* (38). A disruption of the *SIR3* gene, *sir3*::*LEU2*, that abrogates silencing of *HML* transcription was also examined (38). Sir3p functions in transcriptional silencing via binding Sir4p, Rap1p, and histones to condense chromatin structure (13, 30, 68). We also examined two ORC mutants, *orc5-1,3* and *orc5-1,2*, which are competent for the initiation of DNA replication but defective in transcriptional silencing (25).

The parental strain used for construction of the E and I silencer mutations and the *sir3* mutation was used as a control. In the wild-type parental strain, the *HML* ARS cluster containing *ARS302*, *ARS303*, and *ARS320* shows a Y arc indicative of passive replication, but no bubble arc (Fig. 4A). Thus, no replication origin activity is detected at the *HML* ARS cluster in the parental strain, a finding consistent with the results obtained above in other wild-type strains. Also, Y arcs, but no bubble arcs, were seen in strains with *trans*-acting mutations in *sir3* (Fig. 4B), *orc5-1,2* (not shown), *orc5-1,3* (Fig. 4E), or *cis*-acting mutations that delete either the I silencer alone (Fig. 4C) or both the E and I silencers (Fig. 4D). Thus, in all of the strains tested, *ARS303* and *ARS320* remain inactive as replication origins. In addition, when present, *ARS302* also remains inactive (Fig. 4A, B, and E). Several of these mutations are known to abrogate transcriptional silencing at *HML* (Fig. 4, *HML*$^+$). Also, mutations in the *SIR1* or *SIR4* genes that abrogate transcriptional silencing at *HML* showed no replication origin activity at *ARS302* in the chromosome (20). Thus, in a variety of strains with mutations that derepress transcription at *HML*, the ARSs *ARS303*, *ARS320* and, when present, *ARS302* are silent as replication origins.

Replication origin silencing at the *HML* ARS cluster is unaffected by special growth conditions that induce expression of the neighboring *CHA1* gene. Replication origins are sometimes located near genes that are transcriptionally active or whose transcription is induced by special growth conditions or during development (4, 36). One possibility is that the normally silent origins at the *HML* ARSs are specialized replication origins that are activated in the growth conditions that result in the induction of expression at the neighboring gene, *CHA1* (catabolism of hydroxy amino acids). *CHA1* encodes the enzyme L-serine (L-threonine) dehydratase, which permits growth of *S. cerevisiae* in conditions where the hydroxy amino acids are the only source of nitrogen (5). In such a growth condition, *CHA1* mRNA levels are among the most abundant of all chromosome III transcripts (65), while in standard growth conditions the mRNA is not detectable (52). The *CHA1* gene is normally transcriptionally repressed, and the special growth conditions induce *CHA1* expression by disrupting a positioned nucleosome which occludes the promoter region for gene transcription (44).

To test for possible replication origin activation at *HML* ARS cluster in this special growth condition, replication intermediates were isolated from *S. cerevisiae* X2180-B (*MAT*α) grown in synthetic minimal media without or with L-serine as the sole nitrogen source. Northern blot analysis confirmed the induction of *CHA1* mRNA (data not shown). 2-D gel electrophoresis analysis of DNA replication intermediates showed a Y arc but no bubble arc at the *HML* ARS cluster region containing *ARS302*, *ARS303*, and *ARS320*, indicating that this

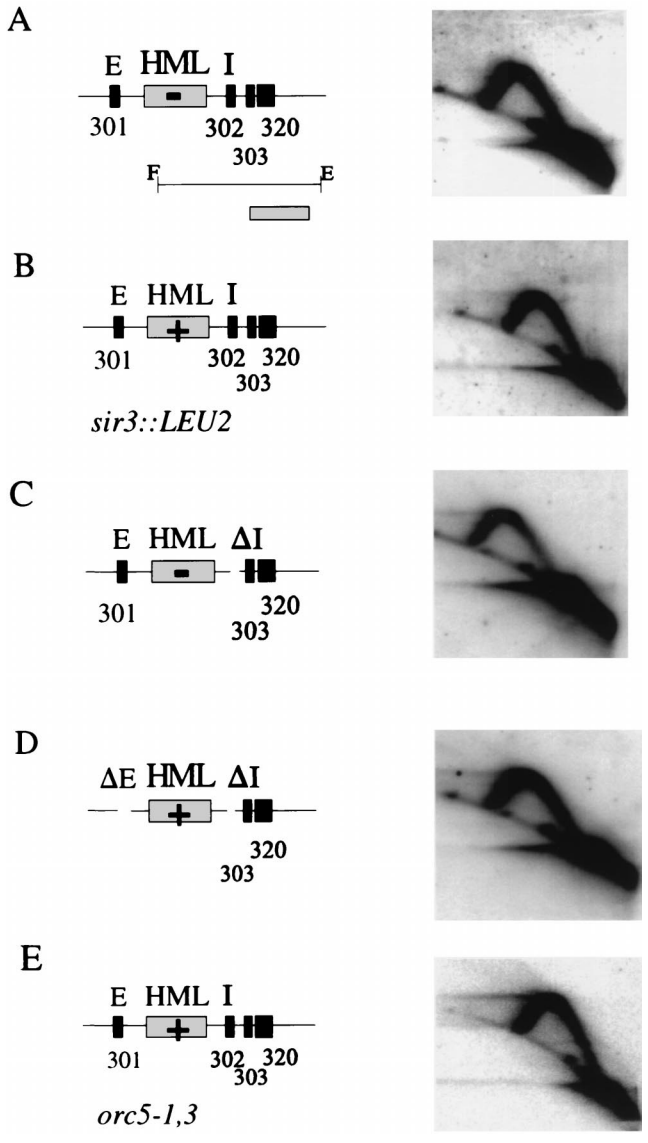

FIG. 4. Origin inactivity at the *HML* ARS cluster is independent of mutations that abrogate transcriptional silencing. Five different strains were used to analyze chromosomal origin activity at the *HML* locus. The maps in all panels show the position of the *HML* locus, the E and I silencer elements, the ARS elements, and the transcriptional status of *HML* (−, silent; +, active). (A) DMY1, the parental strain. (B) DMY2, strain with a mutation disrupting the *SIR3* gene (shown as *sir3*::*LEU2*). (C) DMY94, strain with the I silencer deleted (ΔI). (D) DMY95, strain with E and I silencers deleted (ΔEΔI). (E) JRY4556, strain with a mutation in *ORC5* (*orc5-1,3*) which is defective in transcriptional silencing but competent in replication. Replication intermediates within the 3.9-kb *Fsp*I/*Eco*RI fragment (see map in panel A) were detected by using the *Hin*dIII/*Bam*HI fragment as a probe (shown as hatched box in panel A). 2-D gels show no replication bubble at *HML ARS303*, *ARS320*, and *ARS302* (where present) in all five strains. *HML*, silent mating-type locus; E and I, silencer elements; 303, *ARS303*; 320, *ARS320*; 302, *ARS302*; 301, *ARS301*.

region remains inactive as a replication origin in this special growth condition (Fig. 5B, *HML* ARS cluster). Similar results were seen for an isogenic *MAT***a** strain (X2180-A; data not shown). The absence of detectable replication bubbles at the *HML* ARS cluster is not due to their artifactual breakage under the special growth conditions of *CHA1* induction, since the same membrane reprobed to detect replication intermediates at ORI305 shows a strong bubble-arc signal (Fig. 5B, ORI305). We conclude that the *HML* ARS cluster is not used as a replication origin under the special growth conditions required for induction of the neighboring *CHA1* gene.

Activation of silent origins in the *HML* ARS cluster after deletion of replication origins at remote locations in the chromosome. The *HML* region is duplicated by replication forks that originate from early firing origins at remote locations in chromosome III (54). ORI305 is the origin closest to *HML* (Fig. 6A, WT) and is one of the earliest firing origins on chromosome III. ORI305 is a highly efficient replication origin, and 2-D gel analysis shows that the origin fires in practically every S phase in a variety of yeast strains (31, 34; the present study). The next closest origin in the chromosome is ORI306 (Fig. 6A, WT), which is also early firing and highly efficient (54, 71). Inactivation of the closest origins is expected to move the source of replication forks farther away from *HML*, increasing the time it takes for replication forks to reach the *HML* ARS cluster. We wondered whether inactivating ORI305 and ORI306 would affect replication origin silencing at *HML*.

Deletion of ORI305 or ORI306, resulting in the strains Δ305 or Δ306, respectively, is known to inactivate replication origin function at the respective loci (18, 31). We first tested whether inactivating ORI305 and ORI306 in this way affected origin silencing at the *HML* ARS cluster. Replication intermediates

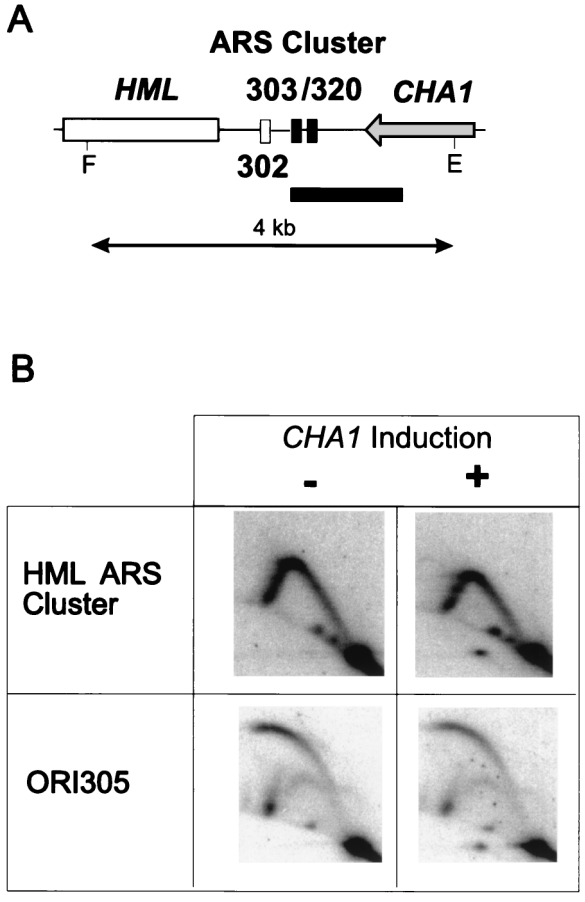

FIG. 5. Effect of *CHA1* induction on chromosomal replication activity of the *HML* ARS cluster. Replication activity at the *HML* ARS cluster region was analyzed in special growth conditions where expression of the neighboring *CHA1* gene was induced by more than 100 fold. (A) Map of relevant locus. *Hin*dIII/*Bam*HI fragment used as a probe in 2-D gel analysis is shown as black box below the map. (B) 2-D gel analysis of replication intermediates at the *HML* locus and at ORI305 (positive control for detection of replication bubble). F, *Fsp*I; E, *Eco*RI.

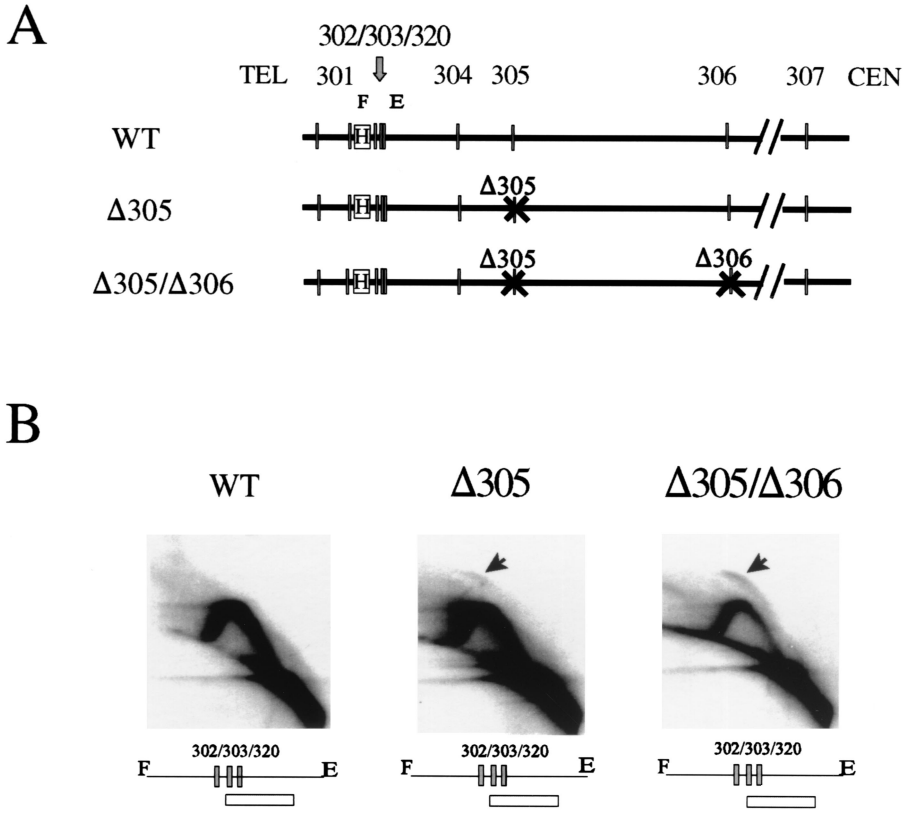

FIG. 6. 2-D gel analysis of the *HML* ARS cluster in *S. cerevisiae* strains with or without deletion of the closest replication origins. *ARS303*, *ARS320*, and *ARS302* were analyzed by 2-D electrophoresis in a wild-type (WT) strain and in two mutant strains deleted for ORI305 (Δ305) or both ORI305 and ORI306 (Δ305/Δ306). (A) Map with the details of the left portion of chromosome III in each strain. The box marked "H" represents the *HML* locus. (B) 2-D gel analysis of all three strains. The same restriction digest, *Fsp*I/*Eco*RI, was used as in previous experiments (see the maps below each column), producing a 3.9-kb fragment at the *HML* locus. The *Hin*dIII/*Bam*HI fragment (1.4 kb) was used as the probe (shown as an open box below the maps). Arrows in 2-D gels point to a bubble arc resulting from replication origin activity at the *HML* ARS cluster.

were isolated from the parental strain (Fig. 6A, WT) and two mutant strains (Fig. 6A, Δ305 and Δ305/Δ306), and then the DNA was digested with *Fsp*I and *Eco*RI and subsequently analyzed by 2-D gel electrophoresis. In mutant strains where one or two active origins were deleted, replication initiation occurred at the *HML* locus, as evidenced by the detection of bubble arcs (Fig. 6B, Δ305 and Δ305/Δ306). Upon close inspection, a bubble arc is just barely detectable in the strain Δ305 with ORI305 deleted. The frequency of initiation was greater in the strain deleted for both ORI305 and ORI306 (Δ305/Δ306) compared to the strain deleted for ORI305 alone, as evidenced by a stronger bubble arc signal accompanied by a weaker Y-arc signal. The origin at the *HML* ARS cluster functions at a low level, as indicated by the low ratio of bubble arc to early-Y-arc signals. The results show that replication origin function is clearly detectable at the *HML* ARS cluster after the deletion of ORI305 and ORI306, two known early-firing origins that are the closest to *HML* in the chromosome.

A silent origin at *ARS301*, but not at *ARS304*, is activated after deletion of active origins at remote locations in the chromosome. We also tested whether *ARS301* and *ARS304* on the left arm of chromosome III (see map in Fig. 1) would be active replication origins at their native locations after deletion of ORI305 and ORI306. Both *ARS301* and *ARS304* are active on a plasmid but are inactive as replication origins at their native locations in the chromosome (14, 20). Paradoxically, *ARS301* is known to form a prereplication complex in the chromosome (58), despite its inactivity as an origin. Genomic DNA from all three strains of yeast (WT, Δ305, and Δ305/Δ306) was isolated, digested with *Fsp*I (for *ARS301*) and *Fsp*I/*Bgl*I (for *ARS304*), and analyzed by 2-D gel electrophoresis.

ARS301, the *HML* E silencer, showed no detectable bubble arc in the strain where only ORI305 was inactivated by deletion (Fig. 7A, Δ305 strain), in contrast to a barely detectable bubble arc seen at the *HML* ARS cluster (Fig. 6B). However, when both ORI305 and ORI306 were deleted, replication origin activity was detected within the genomic DNA fragment containing *ARS301*, as evidenced by the appearance of a bubble arc (Fig. 7A, Δ305/Δ306 strain, arrow). The origin functions at low levels, as indicated by the low ratio of bubble arc to early-Y-arc signals.

ARS304 is located more than 10 kb from *HML*, in between *HML* and ORI305 (Fig. 6A, maps). No detectable replication origin activity was associated with *ARS304* in the parental strain (Fig. 7B, WT) or when ORI305 was deleted (Fig. 7B, Δ305). In contrast to the *HML* ARS cluster and to *ARS301*, no origin activity was seen at *ARS304* in the mutant strains even when both ORI305 and ORI306 were deleted (Fig. 7B, Δ305/Δ306). In all three strains, only Y arcs are seen, indicating that *ARS304* is passively replicated. No traces of bubble arcs are detected even after gross overexposure of the Y-arc signals (data not shown). *ARS304* may be inactive as an origin in a chromosome, or it may be silenced by a mechanism that differs from the mechanism that silences the *HML* ARS cluster and *ARS301*.

Overall, our results show that silent origins at the *HML*

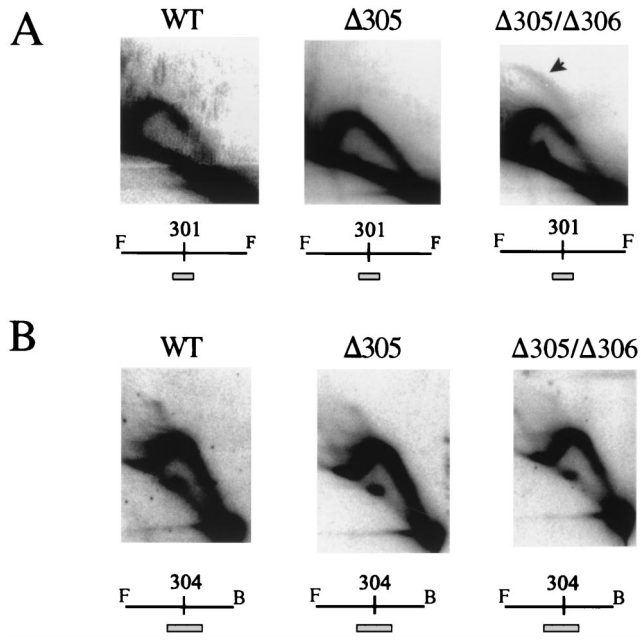

FIG. 7. 2-D gel analysis of *ARS301* and *ARS304* regions in *S. cerevisiae* strains with or without deletion of the closest replication origins. Replication intermediates at silent origins at *ARS301* and *ARS304* in chromosome III were analyzed by 2-D gel electrophoresis. (A) 2-D gel analysis of the *ARS301* region in three different strains (columns marked WT, Δ305, and Δ305/Δ306). Genomic DNA was digested with *Fsp*I producing a 3.9-kb genomic fragment containing *ARS301* (shown on the maps below each 2-D gel picture). The *Sac*I/*Eco*RI fragment (463 bp) was used as a probe (shown as a box below the maps). The arrow indicates the bubble arc resulting from the initiation of replication within the fragment. (B) 2-D gel analysis of *ARS304* region in same three strains of yeast. *Fsp*I and *Bgl*I restriction enzymes were used to generate the 3-kb genomic fragment containing *ARS304* in a central position (maps below each 2D gel). The 0.8-kb *Xba*I/*Pac*I fragment was used as a probe (shown as a box below the maps). No bubble arcs could be detected in this analysis, indicating that *ARS304* does not become detectably active under these experimental conditions.

locus, including the *HML* ARS cluster and *ARS301*, function as active replication origins after deletion of two known early-firing origins from remote locations in the chromosome. In contrast, no origin activity was detectable at *ARS304* in the chromosome.

DISCUSSION

The inactivity of various *HML* ARSs as DNA replication origins in the chromosome has long been puzzling since ARS elements are active replication origins in plasmids. Here we localized two *HML* ARSs, *ARS303* and the newly discovered *ARS320* (Fig. 1). These ARSs exhibited no detectable replication origin activity at their native location between the *HML* locus and the *CHA1* gene (Fig. 2). However, we showed that these ARSs are in fact competent as replication origins in the chromosome when inserted at a position remote from their native location (Fig. 3). These are the first *HML* ARSs shown to exhibit replication origin activity in a chromosome. The results suggested that the replication origin activity of these ARSs is somehow silenced at the *HML* locus.

The locations of the ARSs near the transcriptionally silent *HML* locus, coupled with the fact that transcriptional silencers and replication origins share common functional components, including ARSs and ORC, kept open the possibility that the normal inactivity of these replication origins was linked to the complex pathway for transcriptional silencing of the mating-type locus *HML*. However, we found no replication origin activity at the *HML* ARS cluster regardless of the *trans*-acting mutations in the *SIR3* or *ORC5* genes that abrogate the silencing of transcription (Fig. 4). These findings on *trans*-acting mutations extend those of Dubey et al. (20), who showed that *ARS301* and *ARS302* are not detectably active as origins in *sir1* or *sir4* mutants which abrogate transcriptional silencing. Sir3p was of particular interest here because it exhibits structural similarities with two proteins involved in replication initiation, Cdc6p, as well as with the largest subunit of ORC, Orc1p (3). One hypothesis was that ORC is involved in a mutually exclusive interaction with either Sir3p or Cdc6p and the particular interaction determines whether *HML* ARSs function in transcriptional silencing or in replication initiation. Along this same line of reasoning, the *orc5-1,3* mutant was also of interest because it is competent for initiation of DNA replication but defective in transcriptional silencing (19, 25). However, the absence of detectable replication origin activity at the *HML* ARS cluster in either *sir3* or *orc5-1,3* mutants renders such a hypothesis less likely (Fig. 4). Furthermore, *cis*-acting mutations which delete both the E and I transcriptional silencer elements had no effect on origin silencing at *HML ARS303* and *ARS320*. Deletion of the E and I silencers removes *ARS301* and *ARS302* along with the DNA binding sites for the silencer proteins ORC, Rap1p, and Abf1p, abrogating transcriptional silencing at *HML* (38). In addition to ARSs and ORC, Rap1p and Abf1p can be components of active replication origins in other contexts (41). Our findings suggest that origin silencing at *ARS303*, *ARS320* and, where present, *ARS302* is independent of transcriptional silencing, despite the fact that replication origins and transcriptional silencers share key *cis*- and *trans*-acting components.

Other plausible hypotheses for silencing of replication origins near *HML* include an unfavorable nuclear localization or inaccessibility of the DNA in a heterochromatin-like structure. *HML* is located near the left telomere of chromosome III which is tethered to the nuclear periphery along with Sir3p (51), a component of the heterochromatin-like structure at and near *HML* (30). DNA in *HML* is highly inaccessible to exogenous factors such as enzymes (63). In mutant strains disrupted in *SIR3*, telomeres lose their perinuclear localization, and DNA in *HML* becomes accessible in a more open chromatin structure. Despite these marked changes in *HML* nuclear localization and accessibility known to accompany abrogation of transcriptional silencing, we found that *SIR3* disruption and other mutations that abrogate transcriptional silencing, including *orc5-1,3* and deletion of E and I silencers, did not lead to loss of replication origin silencing at *ARS303*, *ARS320* and, where present, *ARS302*.

Another hypothesis is that silent origins are specialized replication origins that function only in some stage in the yeast life cycle or under special growth conditions (21). With respect to meiosis, *HML* ARSs tested which have no origin activity in the chromosome during mitotic S phase are also not detectably active in premeiotic S phase (14). We examined yeast mating type since it is known to have profound effects on DNA recombination in the entire left arm of chromosome III (70). However, no detectable replication origin activity was seen at the *HML* ARS cluster in either *MAT*α or *MAT***a** cell types (Fig. 2). We also examined special cell growth conditions that induce expression of the nearby *CHA1* gene (52, 65) by disrupting a transcriptionally repressive chromatin structure (44). However, we found that the *HML* ARS cluster showed no detectable replication origin activity under the special growth conditions required for *CHA1* induction (Fig. 5). Thus, the hypothesis that silent origins in yeast are specialized replication origins

that function only in some stage in the yeast life cycle or in special growth conditions is currently without support.

Surprisingly, the *HML* ARS cluster and *HML ARS301* exhibited replication origin activity at their natural locations when two replication origins, located 25 kb (ORI305) and 59 kb (ORI306) away, were inactivated by deletion from the chromosome (Fig. 6 and 7A). These data represent the first demonstration of replication origin activity at the *HML* ARS cluster and at *HML ARS301* in their normal chromosomal locations. A preliminary report of the effects of deleting active chromosomal replicators to the left of *MAT* suggested no apparent origin activation at *HML* in a strain carrying a wild-type *S. cerevisiae* chromosome III as a balancer chromosome (48); however, consistent with our findings in a strain with one copy of *S. cerevisiae* chromosome III, low levels of origin activity were detected upon examination of *HML* ARSs in a strain carrying a brewing yeast balancer chromosome (16a). Our findings show that origin silencing at the *HML* locus is mediated by active replication origins located in the chromosome at long distances from *HML*. In animal cells, distal genetic elements can positively affect replication origin activity (1, 37). Our findings show that in yeast cells, genetic elements that comprise chromosomal replication origins can negatively affect detection of replication origin activity at distal replicators.

The ability of the *HML* ARS cluster to function as a chromosomal origin is consistent with our findings that two of the ARSs, *ARS303* and *ARS320*, are competent as replication origins in the chromosome when inserted at a position remote from the *HML* locus, even when the ARSs are clustered together in their natural configuration (Fig. 3). In contrast, the two ARSs are silent as origins at the *HML* locus independent of whether *ARS302*, the I silencer, is present (Fig. 4A) or has been deleted (Fig. 4C and D). These observations, combined with the fact that in the double origin deletion mutant (Δ305/Δ306) origin activation was seen not only at the *HML* ARS cluster but also at the solitary *ARS301*, suggest that clustering of multiple ARS elements is neither necessary nor sufficient for origin silencing. It is interesting in this connection that an *HMR* silencer region, which contains three independent subregions with ARS activity in a narrow region of DNA, is normally active as a chromosomal origin (35).

Replication origins are known to interfere with or dominate over one another when closely spaced, at distances of less than ~7 kb, resulting in the reduction in activity of both origins or the elimination of activity of one of the two origins (9, 40). The mechanisms for interference and dominance between closely spaced origins are unknown. It is possible that the close spacing of the *HML* ARS cluster with respect to *ARS301* in the chromosome (Fig. 1, chromosome map) may contribute to the reduced origin activity seen at both loci in the Δ305/Δ306 strain, where these ARSs are associated with active replication origins. Other possible explanations include a low intrinsic activity of some of the replicators as seen in ARS assays (Fig. 1) or inhibition of origin activity by flanking sequences in the chromosome (62).

Despite the absence of detectable origin activity at *HML ARS301* in wild-type strains containing ORI305 and ORI306, a pre-RC that includes ORC is known to form in the chromosome (58). Prior to our work, pre-RC formation at the silent *ARS301* origin in a chromosome presented a paradox. The existence of a pre-RC usually reflects a potentially active origin, but the available evidence suggested that *ARS301* was not active as a chromosomal replication origin (20). Our findings here help to resolve this apparent paradox by showing that *ARS301* at its native chromosomal location can be active as a DNA replication origin after deletion of ORI305 and ORI306, suggesting that the *ARS301* pre-RC is competent for origin firing in this condition. In this regard, the properties of *ARS301*, the E silencer at *HML*, begin to resemble more closely the E and I silencers at *HMR* which are competent for origin firing (56, 57).

What is the mechanism for origin silencing at *HML*? The *HML* region is duplicated by replication forks that originate from early-firing origins in chromosome III (54). If the *HML* region was passively replicated before the silent origins could fire, the controls that restrict origin firing to once per S phase would then prevent the *HML* ARSs from functioning as origins during completion of that S phase. The early-firing active origins include ORI305, ORI306, and ORI307 (see maps in Fig. 6A). Deletion of ORI305 and ORI306 makes ORI307 the closest origin to *HML*. The possibility that chromosome III sequences that do not have ARS function in a plasmid become active as replication origins and function efficiently is unlikely since deletions of known origins associated with ARSs have failed to reveal any cryptic origins in the left arm of chromosome III (17, 18, 31, 48). *ARS304*, the only remaining ARS between ORI307 and the *HML* ARS cluster, is not active as an origin in wild-type cells (Fig. 7B, WT) (14). Importantly, we found that *ARS304* is not activated as an origin as a result of deleting ORI305 and ORI306 (Fig. 7B, Δ305/Δ306). Thus, deletion of ORI305 and ORI306 likely moves the source of replication forks that duplicate *HML* from 25 kb (ORI305) to 93 kb (ORI307; distances are from *HML ARS303*). Such a large increase in distance necessitates a large delay in time that forks arrive at *HML*. Thus, based on our findings and on information regarding the positions of origins and ARSs on chromosome III, as well as the timing of origin firing (49, 54), we propose the following model to account for the activation of silent origins: a delay in the arrival of forks at *HML* caused by deletion of the closest, early-firing origins relieves origin silencing by permitting additional time for *HML* ARSs to fire as origins. A likely possibility is that *HML* ARSs require additional time because they are activated late in S phase (*ARS301*, see below). Another possibility is that certain *HML* ARSs require more time to fire because they are intrinsically inefficient as replicators (Fig. 1, *ARS303* and *ARS320*). The latter possibility would appear not apply to *ARS301*, which is known to function efficiently as a replicator in a plasmid (58).

The fact that low-level origin activation was seen at the *HML* ARS cluster, as well as at *HML ARS301*, after deletion of ORI305 and ORI306 suggests that the basis for origin silencing at all of the *HML* ARSs may be similar and independent of the intrinsic replicator efficiency in plasmids. *ARS301* is known to fire late in S phase in a plasmid (6). Late-S-phase activation of the *ARS301* origin in the chromosome would be consistent with the model for activation of silent origins proposed above. Also consistent is the fact that every ARS functions as a replication origin in a plasmid. Plasmids, unlike the chromosome, have no additional yeast replication origins and thus no replication forks to duplicate the ARS before it fires as an origin. Finally, the proposed model is consistent with our finding that *ARS303* and *ARS320* can be active replication origins in the chromosome when inserted in place of ORI305, which is located in an early-firing, chromosomal context (23, 54). Our discovery that silent origins can be active as origins under certain conditions opens the possibilities of testing the proposal that the *HML* ARSs are late-activated origins at their natural locations and of identifying the molecular mechanism for origin silencing in a eukaryotic chromosome.

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